DakoCytomation

CE

EGFR pharmDx™

Code K1494

50 tests for use on the Dako Autostainer

Intended use

For In Vitro diagnostic use.

The EGFR pharmDx™ assay is a qualitative immunohistochemical (IHC) kit system to identify epidermal growth factor receptor (EGFR) expression in normal and neoplastic tissues routinely-fixed for histological evaluation. EGFR pharmDx specifically detects the EGFR (HER1) protein in EGFR-expressing cells.

EGFR pharmDx is indicated as an aid in identifying colorectal cancer patients eligible for treatment with ERBITUX™ (cetuximab).

Summary and explanation

Introduction

Epidermal growth factor receptor is a 170 kD transmembrane receptor encoded by the human *HER1* gene. The EGFR protein contains an extracellular ligand binding domain, a transmembrane region and an intracellular domain with intrinsic protein-tyrosine kinase activity. EGFR is homologous to other members of the EGF receptor/erbB family including HER2/erbB2 or neu, HER3/erbB3 and HER4/erbB4.[1,2] The EGFR protein is expressed by a variety of normal cells including many epithelial cell types and tumors derived from them.[3-9] Non-epithelial cell types that express EGFR include smooth muscle, fibroblasts, and nerve.[10]

Specificity

Mouse monoclonal anti-human EGFR, clone 2-18C9 is provided as a hybridoma tissue culture supernatant produced from a mouse immunized with EGFR immunoprecipitated from the A431 cell line (human epidermoid carcinoma). Epitope mapping studies indicate that the antibody recognizes an epitope in the extracellular cysteine-rich region of the molecule spanning sub-domain S2 and proximal to the transmembrane domain. Further mapping of critical amino acids indicates that the epitope is conformational and dependent on disulfide bridging in the native molecule.[11] This monoclonal antibody clone has been shown not to cross react with HER2, HER3, or HER4 and the vector tag, myc. Cytoplasmic staining is commonly seen; however, the test should be repeated if significant cytoplasmic staining makes it difficult to distinguish the diagnostic membrane staining and interpret the results.

Principle of Procedure

The EGFR pharmDx IHC kit system contains reagents required to complete an IHC staining procedure for routinely-fixed, paraffin-embedded specimens. Following incubation with the primary monoclonal antibody, clone 2-18C9, to human EGFR protein, this kit employs a ready-to-use visualization reagent based on dextran technology. This reagent consists of both secondary goat anti-mouse antibody molecules and horseradish peroxidase molecules linked to a common dextran polymer backbone, thus eliminating the need for sequential application of link antibody and peroxidase conjugate. The enzymatic conversion of the subsequently added chromogen results in formation of a visible reaction product at the antigen site. The specimen may then be counterstained and coverslipped. Results are interpreted using a light microscope. Control slides containing two formalin-fixed, paraffin-embedded human cell lines with staining intensity scores of 2+ and 0 are provided for quality control of the kit reagent performance.

Reagents

Code K1494 is for Automated Staining using the Dako Autostainer.

The materials listed are sufficient for 50 tests (50 slides incubated with Primary Antibody to EGFR Protein and 50 slides incubated with the corresponding Negative Control Reagent). The number of tests is based on the use of the EGFR pharmDx Autostainer protocol with all ready-to-use reagents.

The kit provides materials sufficient for a maximum of 10 individual staining runs.

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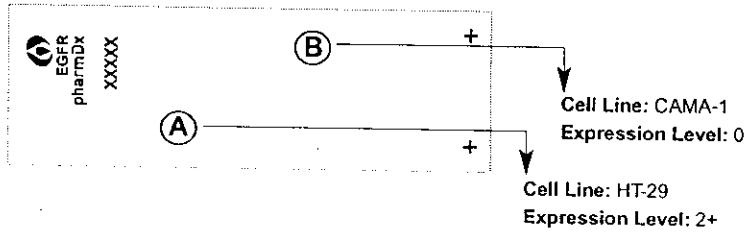
Materials provided	*Quantity*	*Description*
	2x11 mL	**Proteinase K** **PROTEINASE K READY-TO-USE** < 0.1% Proteinase K proteolytic enzyme diluted in a Tris-HCl buffer containing 0.015 mol/L sodium azide.
	2x11 mL	**Peroxidase Block** **PEROXIDASE BLOCK** 3% hydrogen peroxide.
	1x12 mL	**EGFR pharmDx Monoclonal Mouse IgG_1 Antibody** **EGFR pharmDx MOUSE ANTI-HUMAN EGFR PROTEIN** Monoclonal mouse anti-human EGFR (clone 2-18C9) IgG_1 antibody tissue culture supernatant in a Tris-HCl buffer, containing stabilizing protein and 0.015 mol/L sodium azide.
	1x11 mL	**Mouse IgG_1 Negative Control Reagent** **EGFR pharmDx NEGATIVE CONTROL REAGENT** Monoclonal mouse IgG_1 antibody tissue culture supernatant in a Tris-HCl buffer, containing stabilizing protein and 0.015 mol/L sodium azide.
	2x11 mL	**Labelled Polymer, HRP** **LABELLED POLYMER-HRP** Dextran polymer conjugated with horseradish peroxidase and affinity-isolated goat anti-mouse immunoglobulins. Supplied in Tris-HCl buffer containing stabilizing protein and an antimicrobial agent.
	10x11 mL	**DAB+ Substrate Buffer** **DAB+ SUBSTRATE BUFFER** Substrate buffer solution, pH 7.5, containing <0.1% hydrogen peroxide, stabilizers, enhancers and an antimicrobial agent.
	2x3 mL	**Liquid DAB+ Chromogen** **DAB+ CHROMOGEN** 5% 3,3'-diaminobenzidine in chromogen solution.
	2x1 L	**Wash Buffer 10x** **WASH BUFFER (10X)** Tris buffered saline solution containing Tween 20, pH 7.6.
	2x5 slides	**EGFR pharmDx™ Control Slides** **EGFR pharmDx CONTROL SLIDES** Each slide contains sections of two pelleted, formalin-fixed, paraffin-embedded human cell lines, which represent a moderate level of EGFR protein expression and no EGFR expression. The IHC staining scores of the cell pellets are 2+ and 0.



Materials required, but not supplied

Ammonium hydroxide, 15 mol/L diluted to 0.037 mol/L
Counterstain: Hematoxylin, alcohol or water-based such as DakoCytomation's Hematoxylin (code S3302)
Coverslips
Distilled or deionized water (reagent-quality water)
Drying oven, capable of maintaining 56–60°C
Ethanol, absolute and 95%
Light microscope (4x–40x objective magnification)
Mounting medium, such as DakoCytomation's Faramount (code S3025)
or DakoCytomation's Glycergel (code C0563) or DakoCytomation's Ultramount (code S1964)
Positive and negative tissues to use as process controls (see Quality control section)
Slides, Fisher's SuperFrost Plus, poly-L-lysine-coated slides, charged slides,
or DakoCytomation's Silanized Slides (code S3003) (see Specimen preparation section)
Staining jars or baths
Timer (capable of 2–30 minute intervals)
Wash bottle
Xylene, toluene, or xylene substitutes
1 mL pipette
Dako Autostainer Universal Staining System (code S3400) or Autostainer Plus (code S3800) with Dako Autostainer software version 3.0.0 or higher. (Please refer to the Dako Autostainer User Guide for necessary components.)

Note: All reagents included or available separately such as DakoCytomation's Wash Buffer (code S3006) are formulated specifically for use with this test. For the test to perform as specified, no substitutions can be made.

Specimen preparation

Biopsy specimens must be handled to preserve the tissue for IHC staining. Standard methods of tissue processing should be used for all specimens.[13]

Specimens preserved in the following fixatives are suitable for testing with EGFR pharmDx: 10% (v/v) neutral buffered formalin, 10% (v/v) unbuffered formalin, 25% (v/v) unbuffered formalin, AFA (acetic formalin alcohol), Richard-Allen Scientific's Pen-fix and Bouin's fixative. Tissues fixed in Anatech's PreFer are not suitable for EGFR pharmDx testing. Use of EGFR pharmDx on PreFer fixed tissues results in unsatisfactory preservation of morphology and may cause erroneous results.[14]

Paraffin-embedded sections
Routinely processed and paraffin embedded tissues are suitable for use. Specimens from the biopsy should be blocked into a thickness of 3 or 4 mm and fixed for the time period appropriate to the fixative. The tissues are then dehydrated and cleared in a series of alcohols and xylene, followed by infiltration by melted paraffin. The paraffin temperature should not exceed 60°C. Properly fixed and embedded tissue blocks expressing the EGFR protein will keep indefinitely prior to sectioning and slide mounting if stored in a cool place (15–25°C).[13,15]

Tissue specimens should be cut into sections of 3–5 µm. After sectioning, tissues should be mounted on Fisher's SuperFrost Plus, DakoCytomation's Silanized (code S3003), charged slides or poly-L-lysine coated slides and placed in drying racks. The slide racks should be pounded on an absorbent towel to remove water trapped under paraffin and on glass and then dried at room temperature for one hour. The rack of slides should then be placed in a 56–60°C incubator for one hour. Any excess water remaining on slides after removal from the incubator should be removed by pounding slides on towels and drying for one additional hour in the incubator. After removal from the incubator, slides should be held at room temperature until cool and paraffin has hardened. To preserve antigenicity, tissue sections, mounted on slides (Fisher's SuperFrost Plus, poly-L-lysine, charged or DakoCytomation's Silanized slides (code S3003), should be stained within 2 months of sectioning when held at room temperature (20–25°C).[14] Consult the DakoCytomation Handbook: "Immunochemical Staining Methods"[16] or References 13 and 15 for further details on specimen preparation.

The use of this test on decalcified tissues has not been validated and is not recommended. The slides required for EGFR evaluation and verification of tumor presence should be prepared at the same time.

A minimum of 5 slides is recommended, 1 slide for tumor presence, 2 slides for EGFR protein evaluation (one slide for primary antibody and one slide for Negative Control Reagent), and 2 slides for back-up.

Precautions

1. For professional users.
2. This product contains sodium azide (NaN_3), a chemical highly toxic in pure form. At product concentrations, though not classified as hazardous, build-ups of NaN_3 may react with lead and copper plumbing to form highly explosive metal azides. Upon disposal, flush with large volumes of water to prevent azide build-up in plumbing.[17]
3. As with any product derived from biological sources, proper handling procedures should be used for specimens as well as reagents.
4. Minimize microbial contamination of reagents to avoid nonspecific staining.
5. Incubation times, temperatures, or methods other than those specified may give erroneous results.
6. Reagents have been optimally diluted. Further dilution may result in loss of antigen staining.
7. Do not substitute primary antibodies or negative control reagents with primary antibodies and negative control reagents of different manufactured lots (lot numbers appear on vial labels) or with reagents from other manufacturers.
8. The Labeled Polymer, Liquid DAB+ Chromogen and prepared DAB+ Substrate-Chromogen solution may be affected adversely if exposed to excessive light levels. Do not store system components or perform staining in strong light, such as direct sunlight.
9. Wear appropriate Personal Protective Equipment to avoid contact with eyes and skin.[18] Unused solution should be disposed of according to local, State, and Federal regulations.
10. Safety Data Sheet available for professional users on request.

Risk and Safety Statements

DAB Chromogen

R 40	Limited evidence of a carcinogenic effect.
S 36/37	Wear suitable protective clothing and gloves – may cause eye and skin irritation.

Storage

Store EGFR pharmDx kit system at 2–8°C.

Control slides must also be stored at 2–8°C.

Prepared Substrate-Chromogen Solution (DAB) is stable for approximately 5 days when stored at 2–8°C.

Do not use the kit after the expiration date printed on the outside of the kit box. There are no obvious signs to indicate instability of this product, therefore, positive and negative controls should be run simultaneously with patient specimens. If the reagents are stored under any conditions other than those specified in the package insert, they must be validated by the user.[12]

Instructions for use

Reagent Preparation
The following reagents must be prepared prior to staining:

Wash Buffer Solution
Prepare a sufficient quantity of wash buffer by diluting Wash Buffer 10x, 1:10 using distilled or deionized water (reagent-quality water) for the wash steps. Discard buffer if cloudy in appearance.

Substrate-Chromogen Solution (DAB+)
This solution should be mixed thoroughly prior to use. Any precipitate developing in the solution does not affect staining quality.

To prepare DAB+ Substrate-Chromogen Solution, add 11 drops of Liquid DAB+ Chromogen to one vial of DAB+ Substrate Buffer and mix. Discard any unused solution.

Important Note: The color of the Liquid DAB+ Chromogen in the bottle may vary from clear to light lavender-brown. This will not affect the performance of this product. Dilute per the guidelines above. Addition of excess Liquid DAB+ Chromogen to the DAB+ Substrate Buffer will result in deterioration of the positive signal.

Counterstain
Prepare ammonia water for counterstain bluing if required.

Ammonia water (0.037 mol/L) is prepared by mixing 2.5 (±0.5) mL of 15 mol/L (concentrated) ammonium hydroxide with 1 liter of reagent quality water. Unused 0.037 mol/L ammonia water may be stored at room temperature (20–25°C) in a tightly capped bottle for up to 12 months.

Mounting Medium
Mounting media such as DakoCytomation's Faramount Aqueous Mounting Medium, Ready-to-use (code S3025) or DakoCytomation's Glycergel Mounting Medium (code C0563) is recommended for aqueous mounting. Liquify Glycergel by warming to approximately 40(±5) °C prior to use.

Non-aqueous, permanent mounting is also suitable, such as DakoCytomation's Ultramount (code S1964).

Staining procedure on the Dako Autostainer

Procedural Notes
The user should read these instructions carefully and become familiar with all components prior to use (see Precautions). Refer to "Dako Autostainer, Universal Staining System, User Guide".

All reagents should be equilibrated to room temperature (20–25°C) prior to immunostaining. Likewise, all incubations should be performed at room temperature.

Do not allow tissue sections to dry during the staining procedure. Dried tissue sections may display increased nonspecific staining. To avoid drying of tissues, place slides in a humid chamber.

Deparaffinization and Rehydration
Prior to staining, tissue slides must be deparaffinized to remove embedding medium and rehydrated. Avoid incomplete removal of paraffin. Residual embedding medium will result in increased nonspecific staining.

STEP 1. Place slides in a xylene bath and incubate for 5 (±1) minutes. Change baths and repeat once.
STEP 2. Tap off excess liquid and place slides in absolute ethanol for 3 (±1) minutes. Change baths and repeat once.
STEP 3. Tap off excess liquid and place slides in 95% ethanol for 3 (±1) minutes. Change baths and repeat once.
STEP 4. Tap off excess liquid and place slides in reagent-quality water for 5 (±1) minutes.
STEP 5. Tap off excess liquid and place slides in Wash Buffer. Begin staining procedure as outlined in Staining Protocol.

Xylene and alcohol solutions should be changed after 40 slides.

Toluene or xylene substitutes, such as Histoclear, may be used in place of xylene.

EGFR pharmDx includes pretreatment by means of a proteolytic enzyme digestion step. Tissue sections may occasionally be overdigested, causing disruption of cell membranes and overall tissue architecture. Run the assay with careful attention to the duration of the proteolytic digestion step.

Note: If overdigestion is a persistent problem, 10% neutral buffered formalin-fixed tissues may be post-fixed in 10% neutral buffered formalin for 10 minutes after deparaffinization. See procedure below:

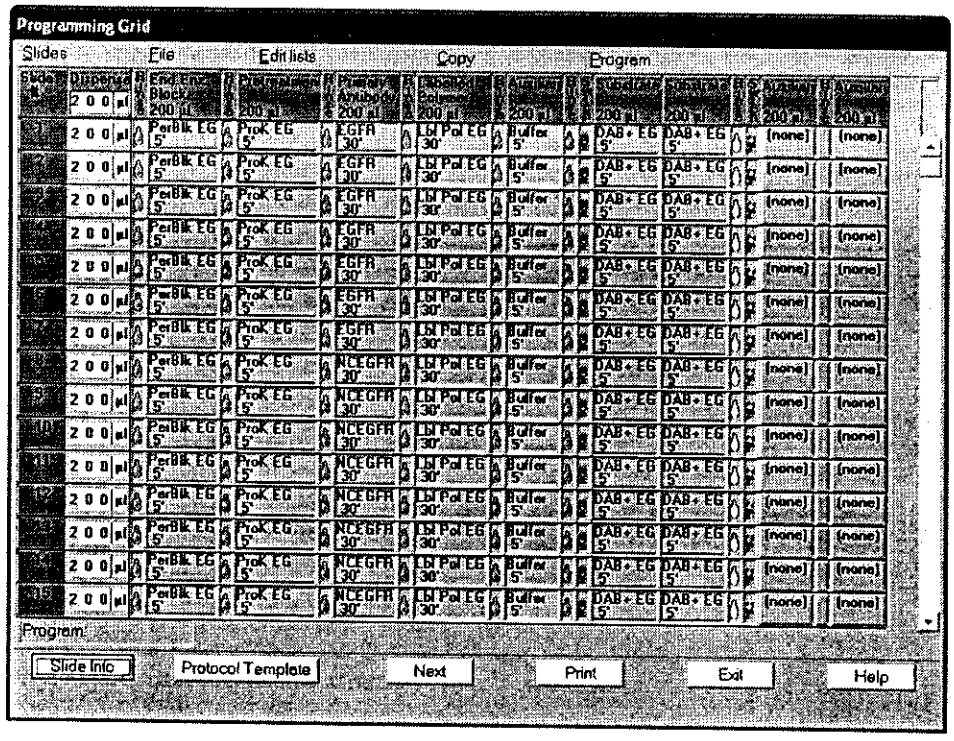
Post-fixation procedure
1. Deparaffinize sections and immerse in reagent quality water.
2. Immerse slides in a 10% neutral buffered formalin for 10 minutes.
3. Rinse slides twice in deionized or distilled water.
4. Continue with the EGFR pharmDx staining procedure.

Automated Staining Protocol
Refer to "Dako Autostainer, Universal Staining System, User Guide".

STEP 1. Select protocol and program staining run.
STEP 2. Use Auto programs to set up program and begin the EGFR pharmDx program.
STEP 3. Place the reagent vials in the Dako Autostainer reagent rack according to the computer generated reagent map.
STEP 4. Load the slides onto the Dako Autostainer according to the computer generated slide map.
STEP 5. Begin the run.
STEP 6. Remove slides from the Dako Autostainer.

Proceed to Counterstain and Mounting.
Note: Rinse slides in reagent-quality water after the DAB+ Substrate-Chromogen solution step.
(Dako Autostainer hardware versions 02 and 03 rinse the slides in reagent-quality water after the substrate-chromogen step. The 01 hardware version of the Dako Autostainer rinses slides in buffer. Therefore, slides that are stained on 01 hardware must be rinsed with the reagent-quality water after they have been removed from the Autostainer).

Figure 1. Autostainer Programming Grid
Representation of the program run on the Dako Autostainer.



The programming grid (above) is a Master Protocol Template containing protocol elements to run all of the detection systems along with the EGFR pharmDx kit. Only EGFR reagents are shown in this example grid.

Counterstain (instructions for Hematoxylin)

The colored end-product of the staining reaction is alcohol and water insoluble. Hematoxylin, either alcohol or water-based such as DakoCytomation's Hematoxylin (code S3302) may be used. Do not use regressive counterstains.

STEP 1. Remove slides from the Dako Autostainer and counterstain in hematoxylin as described below.
STEP 2. Immerse slides in a bath of hematoxylin. Incubate for 2–5 minutes, depending on the strength of hematoxylin used.
STEP 3. Rinse gently in a reagent-quality water bath. Ensure that all residual hematoxylin has been cleared. Optional: Dip slides 10 times into a bath of 0.037 mol/L ammonia water (see Reagent preparation section). The ammonia water step is not required when using hematoxylin.

Note: The use of DakoCytomation's Hematoxylin (code S3302) is strongly recommended. Using a 5-minute incubation, this counterstain produces a mild purple/blue end product that does not obscure specific immunostaining. Strong counterstaining may mask weak EGFR expression.

STEP 4. Rinse *gently* in a reagent-quality water bath for 2–5 minutes.

Mounting

Mounting media such as DakoCytomation's Faramount Aqueous Mounting Medium, Ready-to-use (code S3025) or DakoCytomation's Glycergel Mounting Medium (code C0563) is recommended for aqueous mounting. Liquify Glycergel by warming to approximately 40(±5)°C prior to use.

Non-aqueous, permanent mounting is also suitable such as DakoCytomation's Ultramount (code S1964).

Note: Slides may be read when convenient. However, some fading may occur if slides are coverslipped with an aqueous mounting medium and exposed to strong light. To minimize fading, store slides in the dark at room temperature (20–25°C).

Quality control

Deviations in the recommended procedures for tissue fixation, processing and embedding in the user's laboratory may produce significant variability in results, necessitating regular performance of in-house controls in addition to the DakoCytomation-supplied Control Slides. In the USA, consult the quality control guidelines of the College of American Pathologists (CAP) Certification Program for Immunohistochemistry. See also NCCLS Quality Assurance for Immunocytochemistry Approved Guideline,[19] and References 20–23 for additional information.

EGFR pharmDx Control Slides (provided)
Each of the supplied Control Slides contains two pelleted, formalin-fixed, paraffin-embedded human cell lines with staining intensity scores of 2+ and 0. One slide should be stained in each staining procedure. The evaluation of the DakoCytomation-supplied Control Slide cell lines indicates the validity of the staining run. They

should not be used as an aid in interpretation of patient results.

Positive Control Tissue
Controls should be fresh autopsy/biopsy/surgical specimens fixed, processed and embedded as soon as possible in the same manner as the patient sample(s). Positive tissue controls are indicative of correctly prepared tissues and proper staining techniques. One positive tissue control for each set of test conditions should be included in each staining run.

The tissues used for the positive tissue controls should give weak positive staining so they can detect subtle changes in the primary antibody sensitivity. The Control Slides supplied with this system or specimens processed differently from the patient sample(s) validate reagent performance only and do not verify tissue preparation.

Known positive tissue controls should only be utilized for monitoring the correct performance of processed tissues and test reagents, NOT as an aid in formulating a specific diagnosis of patient samples. If the positive tissue controls fail to demonstrate appropriate positive staining, results with the test specimens should be considered invalid.

Normal cell types that may be used as a weak EGFR positive include glandular epithelial cells of the prostate and normal lung bronchial epithelium. Because the EGFR staining pattern is heterogeneous, weakly staining normal tissue elements can also be negative. A strong positive internal tissue control is perineurium.

Negative Control Tissue
Use a negative control tissue (known to be EGFR protein negative) fixed, processed and embedded in a manner identical to the patient sample(s) with each staining run to verify the specificity of the primary antibody and to provide an indication of specific background staining. The variety of different cell types present in most tissue sections offers internal negative control sites (this should be verified by the user).

If specific staining occurs in the negative control tissue, results with the patient specimens should be considered invalid. Tissue elements which may be used as negative controls include cardiac myocytes of the heart. Pancreatic acinar cells are also EGFR negative, whereas pancreatic bile duct epithelium stains positively. Lymphocytes when present do not stain with EGFR pharmDx and can be used as a negative internal control.

Nonspecific Negative Control Reagent
Use the supplied Negative Control Reagent in place of the primary antibody with a section of each patient specimen to evaluate nonspecific staining and allow better interpretation of specific staining at the antigen site. The incubation period for the Negative Control Reagent should correspond to that of the primary antibody.

Assay Verification
Prior to initial use of an antibody or staining system in a diagnostic procedure, the user should verify the antibody's specificity by testing it on a series of in-house tissues with known IHC performance characteristics representing known positive and negative tissues. Refer to the Quality control procedures previously outlined in this section of the product insert and to the quality control requirements of the CAP Certification Program for Immunohistochemistry and/or NCCLS Quality Assurance for Immunocytochemistry, Approved Guideline.[19] These quality control procedures should be repeated for each new antibody lot, or whenever there is a change in assay parameters. Carcinomas with known EGFR protein staining intensities and negative tissues are suitable for assay verification.

Table 1: The Purpose of Daily Quality Control

Tissue: Fixed and Processed Like Patient Sample	*Specific Antibody & Detection System*	*Background: Non-specific Antibody (Mouse IgG_1 Negative Control Reagent) provided*
DakoCytomation-supplied Control Slide	Controls staining procedure only. The evaluation of the DakoCytomation-supplied Control Slide cell lines indicates the validity of the staining run.	
Positive Control: Tissue or cells containing target antigen to be detected (could be located in patient tissue). The ideal control is weakly positive staining tissue to be most sensitive to antibody or antigen degradation.	Controls all steps of the analysis. Validates reagent and procedures used for EGFR staining.	Detection of nonspecific background staining.
Negative Control: Tissues or cells expected to be negative (could be located in patient tissue or positive control tissue).	Detection of unintended antibody cross-reactivity to cells/cellular components.	Detection of nonspecific background staining.
Patient Tissue.	Detection of specific staining.	Detection of nonspecific background staining.

Interpretation of staining procedure

Slide evaluation should be performed by a pathologist using a light microscope. All assessments are to be made on the tumor region of the specimen. For evaluation of the immunocytochemical staining and scoring, an objective of 10X or 20X magnification is appropriate. Use intact cells for interpretation of staining results; necrotic or degenerated cells often stain nonspecifically.[19]

Positive and negative cell lines are included in each EGFR pharmDx kit to validate staining runs, every time the assay is performed. Appropriate staining of the control cell lines provides evidence that the EGFR pharmDx assay is functioning properly. No membrane staining of the CAMA-1 control cell line (0) and moderate brown complete or incomplete membrane staining in the HT-29 control cell line (2+) indicates that the staining run is valid. If the staining intensity of the positive control cell line is too weak or too strong a false negative or false positive result may be obtained and the test should be repeated. Reference images are available in the EGFR pharmDx Interpretation Guide.

EGFR pharmDx primarily stains cell membranes, demonstrating both complete and incomplete circumferential staining. The immunostaining pattern is frequently heterogeneous, exhibiting various staining intensities within a single neoplasm. Staining has also been observed in the cytoplasm and extracellular spaces.

EGFR pharmDx test results should be reported as positive or negative, using membrane staining as the evaluable structure. Positivity for EGFR expression is defined as any membrane staining above background level, whether or not completely circumferential. Absence of staining should be reported as negative.

Cytoplasmic staining is commonly seen, however the test should be repeated if significant cytoplasmic staining makes it difficult to distinguish membrane staining and interpret the results. Depending on the incubation length and potency of the hematoxylin used, counterstaining will result in a pale to dark blue coloration of the cell nuclei. Excessive or incomplete counterstaining may compromise interpretation of results.

Table 2: EGFR pharmDx Staining Results

Report to treating physician	Definition	
EGFR negative	Absence of specific membrane staining within the tumor	
EGFR positive	Positive (1+) staining is defined as any IHC staining of tumor cell **membranes** above background level; whether it is complete or incomplete circumferential staining.	
	Staining intensity	Percent of tumor cells staining
	1+, 2+ or 3+	≥ 1%

Limitations

General Limitations

IHC is a multistep diagnostic process that requires specialized training in the selection of the appropriate reagents; tissue selection, fixation, and processing; preparation of the IHC slide; and interpretation of the staining results.

Tissue staining is dependent on the handling and processing of the tissue prior to staining. Improper fixation, freezing, thawing, washing, drying, heating, sectioning, or contamination with other tissues or fluids may produce artifacts, antibody trapping, or false-negative results. Inconsistent results may be due to variations in fixation and

embedding methods, or to inherent irregularities within the tissue.

Excessive or incomplete counterstaining may compromise proper interpretation of results.

The clinical interpretation of any positive staining or its absence must be evaluated within the context of clinical presentation, morphology and other histopathological criteria. The clinical interpretation of any staining, or its absence must be complemented by morphological studies and proper controls as well as other diagnostic tests. It is the responsibility of a qualified pathologist who is familiar with the antibodies, reagents and methods used to interpret the stained preparation. Staining must be performed in a certified licensed laboratory under the supervision of a pathologist who is responsible for reviewing the stained slides and assuring the adequacy of positive and negative controls.

Tissues from persons infected with hepatitis B virus and containing hepatitis B surface antigen (HBsAg) may exhibit nonspecific staining with horseradish peroxidase.[24]

Reagents may demonstrate unexpected reactions in previously untested tissues. The possibility of unexpected reactions even in tested tissue groups cannot be completely eliminated due to biological variability of antigen expression in neoplasms, or other pathological tissues.[22] Contact DakoCytomation technical support with documented unexpected reactions.

False-positive results may be seen due to non-immunological binding of proteins or substrate reaction products. They may also be caused by pseudoperoxidase activity (erythrocytes) and endogenous peroxidase activity (cytochrome C).[21]

Note: The reagents and instructions supplied in this system have been designed for optimal performance. Further dilution of the reagents or alteration of incubation times or temperatures may give erroneous or discordant results.

Product Specific Limitations
False-negative results could be caused by degradation of the antigen in the tissues over time. Specimens should be stained within 2 months of mounting of tissues on slides when stored at room temperature (20–25°C).[14,25]

For optimal and reproducible results, the EGFR protein requires proteolytic digestion when tissues are routinely fixed (neutral buffered formalin) and paraffin embedded.

Performance characteristics

Specificity
The EGFR antibody, clone 2-18C9 (2-18C9) has been tested for reactivity against cell lines expressing EGFR, HER2, HER3 and HER4. In Western blots of SKBR3 and A431 cell lysates, 2-18C9 recognized a 170 kD band which is consistent with the known molecular weight of EGFR. Clone 2-18C9 has also been found to recognize the EGFRvIII (145 kD) form of the receptor in immunohistochemistry, flow cytometry and Western blotting of EGFRvIII transfected cell lines. In Western blotting experiments, 2-18C9 was unreactive with HER2 positive CAMA-1 cell lysates, HER3-transformed *E. coli* BL-21 protein extracts and CHO-HER4 transfected cell lysates. Additionally, Chinese Hamster Ovary (CHO) transfectants expressing myc (vector tag), either alone or coexpressed with one of the HER family members, were grown in chamber slides that were formalin-fixed and paraffin-embedded, and stained with anti-myc and 2-18C9. The myc antibody stained all five CHO transfectants, whereas 2-18C9 only stained the CHO cells transfected with HER1.

Clinical trials
Three colorectal carcinoma (CRC) cetuximab drug trials (EMD 62202-007, IMCL CP02-9923 and IMCL CP02-0141) were performed in which DakoCytomation EGFR positive immunohistochemistry (IHC) staining test results were used as one of the criteria for study eligibility. In 2 of 3 studies including the pivotal trial (EMD 62202-007), the threshold for positivity was set at 1+ out of a possible 0 to 3+ for staining intensity and 1% staining of the total tumor cells. This threshold was selected because subgroup analysis of a full range of different thresholds of membrane staining criteria and other differentiating criteria could detect no significant difference in clinical outcome.

Pivotal trial
In the pivotal trial (EMD 62202-007), patients with EGFR pharmDx positive test results were treated with cetuximab in combination with irinotecan or with cetuximab alone. 577 tumor specimens were tested. 474/577 (82%, 95% CI = 78.1%, 86.1%) of the CRC specimens tested were positive for EGFR pharmDx expression. 329 EGFR pharmDx patients were available for 2:1 randomization to the two arms of the pivotal drug trial. In this trial, patients who received irinotecan plus cetuximab achieved a response rate of 50/218 (22.9%, 95% CI = 17.5%, 29.1%). Patients who received cetuximab alone achieved a response rate of 12/111 (10.8%, 95% CI = 5.7%, 18.1%). Only DakoCytomation EGFR positive persons received cetuximab treatment. The response rate for EGFR negative persons is unknown, and therefore cannot be compared. There was no correlation between the degree of tumor response and the percentage of EGFR-positive cells or EGFR-staining intensity. (See Table 3)

Table 3: Cetuximab Pivotal Trial (EMD 62202-007) Response Rates

	Total number of patients tested	*Response Rate# of cases treated with cetuximab and irinotecan*	*Response Rate# of cases treated with cetuximab alone*
EGFR pharmDx +	474*	50/218 (22.9%, 95% CI = 17.5%, 29.1%)	12/111 (10.8%, 95% CI = 5.7%, 18.1%)
EGFR pharmDx -	103	None treated	None treated

*329 Patients were available for 2:1 randomization to the two arms of the drug trial

Response rate was the proportion of patients in the entire study population with a decrease by >/=50% in the sum of the perpendicular diameter of all measurable tumor (i.e., a 50% or more decrease in tumor by surface area) that persisted for at least 28 days.

Supportive Studies
The EGFR pharmDx assay was used to enroll patients in the pivotal trial (EMD 62202-007) and one supportive study (IMCL CP02-0141) during cetuximab development. In study IMCL CP02-0141, 140 specimens were tested. Of these specimens, 105 /140 (75%, 95% CI = 66.9%, 83.1%) specimens had tumors that were identified as EGFR pharmDx positive. A total of 61 patients were enrolled in this study; 57 patients received cetuximab.

In an additional supportive study (IMCL CP02-9923), a prototype EGFR pharmDx kit (composed of the same primary antibody and detection system as above), was used to enroll patients. A total of 412 specimens from 401 patients were tested. 292/401 (72.8%, 95% CI = 68.0%, 77.6%) patients had a positive test result. 139 patients were enrolled; 138 received cetuximab plus irinotecan.

Table 4: Summary of EGFR Percent Positivity in Colon Cancer Patients

Study ID	Positive Ratio (# positive/# tested)	% Positive	95% Confidence Intervals
Pivotal Trial EMD 62202-007	474/577	82.1%	78.1 – 86.1%
Supportive Study IMCL CP02-0141	105/140	75.0%	66.9 – 83.1%
Prototype EGFR Study IMCL CP 02-9923	292/401	72.8%	68.0 – 77.6%

Reproducibility
Inter-run reproducibility
Inter-run reproducibility was tested using manual methodology at two laboratories by two technicians in each laboratory over 3 days with 5 different specimens (4 positive, 1 negative in each lab), of different staining intensity scores randomized and masked. Excellent reproducibility (100%) was seen for positive versus negative results (0 vs. 1+, 2+ and 3+). Staining intensity varied by 1+ in two of the positive specimens and by 2+ in one specimen (in one of the tests, the positive tissue element was mostly washed off the slide). The two negative specimens remained negative.

Inter-laboratory reproducibility of staining
Inter-laboratory reproducibility was tested at three geographically separated laboratories with 30 randomized and masked specimens of various IHC staining intensity scores. Freshly cut slides were forwarded to each testing laboratory for manual and automated staining and evaluation by a pathologist. Inter-laboratory percent agreement was 100% for a dichotomous positive/negative determination where 0 was negative and 1+, 2+ and 3+ were positive for EGFR protein expression for both manual and automated testing procedures.

Immunoreactivity
A summary of the EGFR pharmDx immunoreactivity on the recommended panel of normal tissues is presented in Table 5. All tissues were formalin-fixed and paraffin-embedded.

Table 5: Evaluation of Normal Tissue Staining by EGFR pharmDx*

Tissue Type (# tested)	*Positive Tissue Element Staining and Staining Pattern*
Adrenal (2)	Cortical cells (2+): Cytoplasmic
Bone Marrow (3)	None
Breast (2)	Lobular epithelial cells (2+): Membrane and cytoplasmic
Brain/Cerebellum (3)	Molecular layer (1+): Extracellular
Brain/Cerebrum (3)	None
Cervix (3)	Basalar squamous epithelial cells (2+): Membrane
Colon (3)	None
Esophagus (2)	Basalar squamous epithelial cells (2+): Membrane
Heart (3)	None
Kidney (3)	Tubules (1+): Cytoplasmic staining (granular)
Liver (3)	Hepatocytes (sinusoids) (3+); Bile ducts (3+): Membrane and cytoplasmic
Lung (3)	Alveolar lining cells/ basalar bronchial cells (myoepithelial cells) (2+): Membrane and cytoplasmic
Mesothelial Cells (3)	Mesothelial cells (2+): Membrane and cytoplasmic
Ovary (3)	None
Pancreas (3)	Ducts (2+): Membrane
Parathyroid (1)	None
Peripheral Nerve (3)	Nerve cell processes (1+): Fibrous
Pituitary (3)	None
Prostate (3)	Glandular epithelial cells (2+): Membrane
Salivary Gland (3)	Ductal elements (1+): Cytoplasmic
Skeletal Muscle (3)	None
Skin (3)	Squamous cells, adnexal structures (2+): Membrane and cytoplasmic
Small Intestine (3)	None
Spleen (3)	None
Stomach (3)	None
Testis (3)	None
Thymus (3)	None
Thyroid (3)	None
Tonsil (3)	Squamous epithelium (3+): Membrane and cytoplasmic
Uterus (3)	Endometrial gland epithelium (2+): Membrane and cytoplasmic Endometrial stromal cells (2+): Membrane and cytoplasmic Myometrium: None

*The majority of tissues tested had positive staining of fibroblasts in stromal tissue (1+, fibrous) as well as perineural fibroblasts and myoepithelial cells. Endogenous peroxidase-induced staining of eosinophils has been observed occasionally.

Table 6: Troubleshooting

Problem	*Probable Cause*	*Suggested Action*
1. No staining of slides.	1a. Programming error. Reagents not used in proper order.	1a. Check programming grid to verify that the staining run was programmed correctly.
	1b. Reagent vials were not loaded in the correct locations in reagent racks.	1b. Check the Reagent Map to verify the proper location of reagent vials.
	1c. Insufficient reagent in reagent vial.	1c. Ensure that enough reagent is loaded into the reagent vials prior to beginning the run. Refer to Reagent Map for volumes required.
	1d. Sodium azide in wash buffer bath.	1d. Use fresh, azide-free wash buffer provided in the kit.
2. Weak staining of slides.	2a. Inadequate reagent incubation times.	2a. Review Staining Procedure instructions.
	2b. Inappropriate fixation method used.	2b. Ensure that patient tissue is not over-fixed and that an approved fixative is being used. (See Specimen preparation section)
	2c. Overincubation with Proteinase K.	2c. Verify that the Proteinase K solution is incubated for no more than 5 minutes.
3. Excessive background staining of slides.	3a. Paraffin incompletely removed.	3a. Use fresh cleaning solutions and follow procedure outlined in the Deparaffinization and Rehydration section.
	3b. Starch additives used in mounting sections to slides.	3b. Avoid using any additives for adhering sections to glass slides. Many of these are immunoreactive.
	3c. Slides not thoroughly rinsed.	3c. Ensure that the Autostainer is properly primed prior to running. Check to make sure that adequate buffer is provided for entire run. Additional rinse may be applied after the Visualization step, if desired.
	3d. Sections dried during staining procedure.	3d. Verify that the appropriate volume of reagent is being applied to slides. Make sure the Autostainer is run with the hood in the closed position and that it is not exposed to excessive heat or drafts.
	3e. Slides dried while loading the Autostainer.	3e. Ensure slides remain wet with buffer while loading and prior to initiating run.
	3f. Nonspecific binding of reagents to tissue section.	3f. Check for proper fixation of the specimen and/or the presence of necrosis.
4. Tissue detaches from slides.	4a. Use of incorrect slides.	4a. Use charged (Fisher's SuperFrost Plus) or silanized slides such as DakoCytomation's Silanized Slides (code S3003).
5. Excessively strong specific staining.	5a. Inappropriate fixation method used.	5a. Ensure that only approved fixatives and fixation methods are used. (See Specimen preparation section)
	5b. Reagent incubation times too long.	5b. Review Staining Protocol instructions.
	5c. Inappropriate wash solution used.	5c. Use only the diluted wash buffer that is supplied with the kit.
6. Weak staining of the 2+ control slide cell line.	6a. Inadequate Proteinase K proteolytic digestion.	6a. Verify that the Proteinase K solution is programmed for a 5-minute incubation time.
	6b. Inadequate reagent incubation times.	6b. Verify that the incubation times are accurate in programming run.
	6c. Degradation of Control Slide.	6c. Check expiration date and kit storage conditions printed on the package label.
7. Overdigestion of tissue.	7a. Overincubation with Proteinase K.	7a. Verify that the Proteinase K solution is incubated for no more than 5 minutes.
	7b. Inadequate fixation.	7b. Use post-fixation procedure under staining protocol on new slides and stain using the normal procedure.

Note: Refer also to the Troubleshooting section in the DakoCytomation Handbook: Immunochemical Staining Methods, 3rd Edition,[16] the Atlas of Immunohistology,[23] or Immunoperoxidase Techniques. A Practical Approach to Tumor Diagnosis.[20] Contact DakoCytomation Technical Support to report unusual staining.

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N — Dangerous to the Environment French Translation German Translation	O — Oxidizing French Translation German Translation	T+ — Very Toxic French Translation German Translation	Xn — Harmful French Translation German Translation	F+ — Extremely Flammable French Translation German Translation

REF — Catalog / Code Number Reference du catalogue Bestellnummer	Temperature Limitations Limites de temperature Zulassiger temperaturbereich	IVD — *In Vitro* Diagnostic Medical Device Dispositif medical de diagnostic in vitro In-Vitro-Diagnostikum	DakoCytomation, Inc. 6392 Via Real Carpinteria, California 93013 USA Tel 805 566 6655 Fax 805 566 6688 Technical Support 800 424 0021 Customer Service 800 235 5763
Manufacturer Fabricant Hersteller	LOT — Batch Code Code du lot Chargenbezeichnung	Contains Sufficient for <N> Tests French Translation German Translation	EC REP DakoCytomation Denmark A/S Produktionsvej 42 DK-2600 Glostrup Denmark Tel +45 4485 9500 Fax +45 4485 9595 www.dakocytomation.com
Consult *Instructions for Use* Consulter les instructions d'utilisation Gebrauchsanweisung beachten	Use By Utiliser jusque Verwendbar bis	EC REP — Authorized Representative in the European Community French Translation German Translation	

Rev. 02/11/04

DakoCytomation

CE

EGFR pharmDx™

Code K1492
35 tests for manual use

Intended use

For In Vitro diagnostic use.

The EGFR pharmDx™ assay is a qualitative immunohistochemical (IHC) kit system to identify epidermal growth factor receptor (EGFR) expression in normal and neoplastic tissues routinely-fixed for histological evaluation. EGFR pharmDx specifically detects the EGFR (HER1) protein in EGFR-expressing cells.

EGFR pharmDx is indicated as an aid in identifying colorectal cancer patients eligible for treatment with ERBITUX™ (cetuximab).

Summary and explanation

Introduction
Epidermal growth factor receptor is a 170 kD transmembrane receptor encoded by the human *HER1* gene. The EGFR protein contains an extracellular ligand binding domain, a transmembrane region and an intracellular domain with intrinsic protein-tyrosine kinase activity.

EGFR is homologous to other members of the EGF receptor/erbB family including HER2/erbB2 or neu, HER3/erbB3 and HER4/erbB4.[1,2] The EGFR protein is expressed by a variety of normal cells including many epithelial cell types and tumors derived from them.[3-9]

Non-epithelial cell types that express EGFR include smooth muscle, fibroblasts, and nerve.[10]

Specificity
Mouse monoclonal anti-human EGFR, clone 2-18C9 is provided as a hybridoma tissue culture supernatant produced from a mouse immunized with EGFR immunoprecipitated from the A431 cell line (human epidermoid carcinoma). Epitope mapping studies indicate that the antibody recognizes an epitope in the extracellular cysteine-rich region of the molecule spanning sub-domain S2 and proximal to the transmembrane domain. Further mapping of critical amino acids indicates that the epitope is conformational and dependent on disulfide bridging in the native molecule.[11] This monoclonal antibody clone has been shown not to cross react with HER2, HER3, or HER4 and the vector tag, myc. Cytoplasmic staining is commonly seen; however, the test should be repeated if significant cytoplasmic staining makes it difficult to distinguish the diagnostic membrane staining and interpret the results.

Principle of Procedure

The EGFR pharmDx IHC kit system contains reagents required to complete an IHC staining procedure for routinely-fixed, paraffin-embedded specimens. Following incubation with the primary monoclonal antibody, clone 2-18C9, to human EGFR protein, this kit employs a ready-to-use visualization reagent based on dextran technology. This reagent consists of both secondary goat anti-mouse antibody molecules and horseradish peroxidase molecules linked to a common dextran polymer backbone, thus eliminating the need for sequential application of link antibody and peroxidase conjugate. The enzymatic conversion of the subsequently added chromogen results in formation of a visible reaction product at the antigen site. The specimen may then be counterstained and coverslipped. Results are interpreted using a light microscope. Control slides containing two formalin-fixed, paraffin-embedded human cell lines with staining intensity scores of 2+ and 0 are provided for quality control of the kit reagent performance.

Reagents

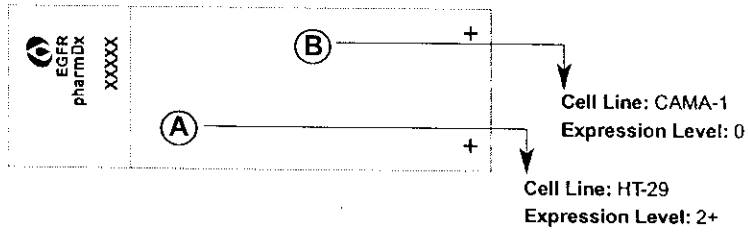
EGFR pharmDx for Manual Use.

The materials listed are sufficient for 35 tests (35 slides incubated with Primary Antibody to EGFR Protein and 35 slides incubated with the corresponding Negative Control Reagent). The number of tests is based on the use of 3 drops of each ready-to-use reagent per slide.

The kit provides materials sufficient for a maximum of 5 individual staining runs.

Materials provided

Quantity	*Description*
1x8 mL	**Proteinase K** **PROTEINASE K READY-TO-USE** < 0.1% Proteinase K proteolytic enzyme diluted in a Tris-HCl buffer containing 0.015 mol/L sodium azide.
1x8 mL	**Peroxidase Block** **PEROXIDASE BLOCK** 3% hydrogen peroxide.
1x4 mL	**EGFR pharmDx Monoclonal Mouse IgG_1 Antibody** **EGFR pharmDx MOUSE ANTI-HUMAN EGFR PROTEIN** Monoclonal mouse anti-human EGFR (clone 2-18C9) IgG_1 antibody tissue culture supernatant in a Tris-HCl buffer, containing stabilizing protein and 0.015 mol/L sodium azide.
1x4 mL	**Mouse IgG_1 Negative Control Reagent** **EGFR pharmDx NEGATIVE CONTROL REAGENT** Monoclonal mouse IgG_1 antibody tissue culture supernatant in a Tris-HCl buffer, containing stabilizing protein and 0.015 mol/L sodium azide.
1x8 mL	**Labelled Polymer, HRP** **LABELLED POLYMER-HRP** Dextran polymer conjugated with horseradish peroxidase and affinity-isolated goat anti-mouse immunoglobulins. Supplied in Tris-HCl buffer containing stabilizing protein and an antimicrobial agent.
1x10 mL	**DAB+ Substrate Buffer** **DAB+ SUBSTRATE BUFFER** Substrate buffer solution, pH 7.5, containing < 0.1% hydrogen peroxide, stabilizers, enhancers and an antimicrobial agent.
1x1 mL	**Liquid DAB+ Chromogen** **DAB+ CHROMOGEN** 5% 3,3'-diaminobenzidine in chromogen solution.
1x1 L	**Wash Buffer 10x** **WASH BUFFER (10X)** Tris buffered saline solution containing Tween 20, pH 7.6.
5 slides	**EGFR pharmDx™ Control Slides** **EGFR pharmDx CONTROL SLIDES** Each slide contains sections of two pelleted, formalin-fixed, paraffin-embedded human cell lines, which represent a moderate level of EGFR protein expression and no EGFR expression. The IHC staining scores of the cell pellets are 2+ and 0.



Materials required, but not supplied

Ammonium hydroxide, 15 mol/L diluted to 0.037 mol/L
Counterstain: Hematoxylin, alcohol or water-based such as DakoCytomation's Hematoxylin (codes S3302) or DakoCytomation's Automation Hematoxylin (code S3301)
Coverslips
Distilled or deionized water (reagent-quality water)
Drying oven, capable of maintaining 56–60°C
Ethanol, absolute and 95%
Humidity chamber
Light microscope (4x–40x objective magnification)
Mounting medium, such as DakoCytomation's Faramount (code S3025) or DakoCytomation's Glycergel (code C0563) or DakoCytomation's Ultramount (code S1964)
Positive and negative tissues to use as process controls (see Quality control section)
Slides, Fisher's SuperFrost Plus, poly-L-lysine-coated slides, charged slides, or DakoCytomation's Silanized Slides (code S3003) (see Specimen preparation section)
Staining jars or baths
Timer (capable of 2–30 minute intervals)
Wash bottle
Xylene, toluene, or xylene substitutes

Note: All reagents included or available separately such as DakoCytomation's Wash Buffer (code S3006) are formulated specifically for use with this test. For the test to perform as specified, no substitutions can be made.

Specimen preparation

Biopsy specimens must be handled to preserve the tissue for IHC staining. Standard methods of tissue processing should be used for all specimens. [13]

Specimens preserved in the following fixatives are suitable for testing with EGFR pharmDx: 10% (v/v) neutral buffered formalin, 10% (v/v) unbuffered formalin, 25% (v/v) unbuffered formalin, AFA (acetic formalin alcohol), Richard-Allen Scientific's Pen-fix and Bouin's fixative. Tissues fixed in Anatech's PreFer are not suitable for EGFR pharmDx testing. Use of EGFR pharmDx on PreFer fixed tissues results in unsatisfactory preservation of morphology and may cause erroneous results.[14]

Paraffin-embedded sections
Routinely processed and paraffin embedded tissues are suitable for use. Specimens from the biopsy should be blocked into a thickness of 3 or 4 mm and fixed for the time period appropriate to the fixative. The tissues are then dehydrated and cleared in a series of alcohols and xylene, followed by infiltration by melted paraffin. The paraffin temperature should not exceed 60°C. Properly fixed and embedded tissue blocks expressing the EGFR protein will keep indefinitely prior to sectioning and slide mounting if stored in a cool place (15–25°C).[13,15]

Tissue specimens should be cut into sections of 3–5 µm. After sectioning, tissues should be mounted on Fisher's SuperFrost Plus, DakoCytomation's Silanized (code S3003), charged slides or poly-L-lysine coated slides and placed in drying racks. The slide racks should be pounded on an absorbent towel to remove water trapped under paraffin and on glass and then dried at room temperature for one hour. The rack of slides should then be placed in a 56–60°C incubator for one hour. Any excess water remaining on slides after removal from the incubator should be removed by pounding slides on towels and drying for one additional hour in the incubator. After removal from the incubator, slides should be held at room temperature until cool and paraffin has hardened. To preserve antigenicity, tissue sections, mounted on slides (Fisher's SuperFrost Plus, poly-L-lysine, charged or DakoCytomation's Silanized slides (code S3003), should be stained within 2 months of sectioning when held at room temperature (20–25°C).[14] Consult the DakoCytomation Handbook: "Immunochemical Staining Methods"[16] or References 13 and 15 for further details on specimen preparation.

The use of this test on decalcified tissues has not been validated and is not recommended. The slides required for EGFR evaluation and verification of tumor presence should be prepared at the same time.

A minimum of 5 slides is recommended, 1 slide for tumor presence, 2 slides for EGFR protein evaluation (one slide for primary antibody and one slide for Negative Control Reagent), and 2 slides for back-up.

Precautions

1. For professional users.
2. This product contains sodium azide (NaN_3), a chemical highly toxic in pure form. At product concentrations, though not classified as hazardous, build-ups of NaN_3 may react with lead and copper plumbing to form highly explosive metal azides. Upon disposal, flush with large volumes of water to prevent azide build-up in plumbing.[17]
3. As with any product derived from biological sources, proper handling procedures should be used for specimens as well as reagents.
4. Minimize microbial contamination of reagents to avoid nonspecific staining.
5. Incubation times, temperatures, or methods other than those specified may give erroneous results.
6. Reagents have been optimally diluted. Further dilution may result in loss of antigen staining.
7. Do not substitute primary antibodies or negative control reagents with primary antibodies and negative control reagents of different manufactured lots (lot numbers appear on vial labels) or with reagents from other manufacturers.
8. The Labeled Polymer, Liquid DAB+ Chromogen and prepared DAB+ Substrate-Chromogen solution may be affected adversely if exposed to excessive light levels. Do not store system components or perform staining in strong light, such as direct sunlight.
9. Wear appropriate Personal Protective Equipment to avoid contact with eyes and skin.[18] Unused solution should be disposed of according to local, State, and Federal regulations.
10. Safety Data Sheet available for professional users on request.

Risk and Safety Statements

DAB Chromogen

R 40	Limited evidence of a carcinogenic effect.
S 36/37	Wear suitable protective clothing and gloves.

Storage

Store EGFR pharmDx kit system at 2–8°C.

Control slides must also be stored at 2–8°C.

Do not use the kit after the expiration date printed on the outside of the kit box. There are no obvious signs to indicate instability of this product, therefore, positive and negative controls should be run simultaneously with patient specimens. If the reagents are stored under any conditions other than those specified in the package insert, they must be validated by the user.[12]

Reagent Preparation

The following reagents must be prepared prior to staining:

Wash Buffer Solution

Prepare a sufficient quantity of wash buffer by diluting Wash Buffer 10x, 1:10 using distilled or deionized water (reagent-quality water) for the wash steps. Discard buffer if cloudy in appearance.

DAB+ Substrate-Chromogen Solution

This solution should be mixed thoroughly prior to use. Any precipitate developing in the solution does not affect staining quality.

The following procedure yields 1 mL of DAB+ Substrate-Chromogen Solution.
STEP 1. Transfer 1 mL of DAB+ Substrate Buffer to a test tube.
STEP 2. Add one drop of Liquid DAB+ Chromogen. Mix and apply to tissue sections with a pipette.

Prepared Substrate-Chromogen Solution (DAB) is stable for approximately 5 days when stored at 2–8°C.

Important Note: The color of the Liquid DAB+ Chromogen in the bottle may vary from clear to light lavender-brown. This will not affect the performance of this product. Dilute per the guidelines above. Addition of excess Liquid DAB+ Chromogen to the DAB+ Substrate Buffer will result in deterioration of the positive signal.

Counterstain

Prepare ammonia water for counterstain bluing if required.

Ammonia water (0.037 mol/L) is prepared by mixing 2.5 (±0.5) mL of 15 mol/L (concentrated) ammonium hydroxide with 1 liter of reagent quality water. Unused 0.037 mol/L ammonia water may be stored at room temperature (20–25°C) in a tightly capped bottle for up to 12 months.

Mounting Medium

Mounting media such as DakoCytomation's Faramount Aqueous Mounting Medium, Ready-to-use (code S3025) or DakoCytomation's Glycergel Mounting Medium (code C0563) is recommended for aqueous mounting. Liquify Glycergel by warming to approximately 40(±5)°C prior to use.

Non-aqueous, permanent mounting is also suitable, such as DakoCytomation's Ultramount (code S1964).

Staining procedure

Procedural Notes

The user should read these instructions carefully and become familiar with all components prior to use (see Precautions section).

All reagents should be equilibrated to room temperature (20–25°C) prior to immunostaining. Likewise, all incubations should be performed at room temperature.

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Do not allow tissue sections to dry during the staining procedure. Dried tissue sections may display increased nonspecific staining. To avoid drying of tissues, place slides in a humid chamber.

Deparaffinization and Rehydration
Prior to staining, tissue slides must be deparaffinized to remove embedding medium and rehydrated. Avoid incomplete removal of paraffin. Residual embedding medium will result in increased nonspecific staining.

STEP 1. Place slides in a xylene bath and incubate for 5 (±1) minutes. Change baths and repeat once.
STEP 2. Tap off excess liquid and place slides in absolute ethanol for 3 (±1) minutes. Change baths and repeat once.
STEP 3. Tap off excess liquid and place slides in 95% ethanol for 3 (±1) minutes. Change baths and repeat once.
STEP 4. Tap off excess liquid and place slides in reagent-quality water for 5 (±1) minutes. Begin staining procedure as outlined in Section C, Staining Protocol.

Xylene and alcohol solutions should be changed after 40 slides.
Toluene or xylene substitutes, such as Histoclear, may be used in place of xylene.

Staining Protocol
STEP 1. **Proteinase K Proteolytic Digestion**
Tap off excess reagent-quality water. Using a lintless tissue (such as a Kimwipe® or gauze pad), carefully wipe around specimen to remove any remaining liquid and to keep reagents within the prescribed area.
Apply enough Proteinase K solution to cover specimen (minimum 3 drops (100 µL)).
Incubate 5 (±0.5) minutes.
Rinse gently with reagent-quality water from a wash bottle (do not focus stream directly on tissue).
Place in a fresh reagent-quality water bath for 5 (±1) minutes.

EGFR pharmDx includes pretreatment by means of a proteolytic enzyme digestion step. Tissue sections may occasionally be overdigested, causing disruption of cell membranes and overall tissue architecture. Run the assay with careful attention to the duration of the proteolytic digestion step.

Note: If overdigestion is a persistent problem, 10% neutral buffered formalin-fixed tissues may be post-fixed in 10% neutral buffered formalin for 10 minutes after deparaffinization. See procedure below:

Post-fixation procedure
1. Deparaffinize sections and immerse in reagent quality water.
2. Immerse slides in a 10% neutral buffered formalin for 10 minutes.
3. Rinse slides twice in deionized or distilled water.
4. Continue with the EGFR pharmDx staining procedure.

STEP 2. **Peroxidase Block**
Tap off excess water and wipe slides as before.
Apply enough Peroxidase Block to cover specimen (minimum 3 drops (100 µL)).
Incubate 5 (±1) minutes.
Rinse gently with Wash Buffer from a wash bottle (do not focus stream directly on tissue).
Place in a fresh Wash Buffer bath for 5 (±1) minutes.
STEP 3. **Primary Antibody or Negative Control Reagent**
Place slides in a humid chamber during the Primary Antibody/Negative Control Reagent and Labeled Polymer incubations to avoid drying of tissues.
Tap off excess buffer and wipe slides as before.
Apply enough Primary Antibody or Negative Control Reagent to cover specimen (minimum 3 drops (100 µL)).
Incubate 30 (±1) minutes in a humid chamber.
Rinse slides as in Step 2.
STEP 4. **Labelled Polymer, HRP**
Tap off excess buffer and wipe slides as before.
Apply enough Labelled Polymer to cover specimen (minimum 3 drops (100 µL)).
Incubate 30 (±1) minutes in a humid chamber.
Rinse slides as in Step 2.
STEP 5. **DAB+ Substrate-Chromogen Solution**
Tap off excess buffer and wipe slides as before.
Apply enough DAB+ Substrate-Chromogen Solution to cover specimen (minimum 3 drops (100 µL)).
Incubate 10 (±1) minutes.
Rinse gently with reagent-quality water from a wash bottle (do not focus flow directly on tissue).
Collect DAB+ Substrate-Chromogen Solution waste in a hazardous materials container for proper disposal.
Place in a reagent-quality water bath for 2–5 minutes.

Counterstain (instructions for Hematoxylin)

The colored end-product of the staining reaction is alcohol and water insoluble. Hematoxylin, either alcohol or water-based such as DakoCytomation's Hematoxylin (codes S3302) or DakoCytomation's Automation Hematoxylin (code S3301) may be used. Do not use regressive counterstains.

STEP 1. Immerse slides in a bath of hematoxylin. Incubate for 2–5 minutes, depending on the strength of hematoxylin used.
STEP 2. Rinse gently in a reagent-quality water bath. Ensure that all residual hematoxylin has been cleared. Optional: Dip slides 10 times into a bath of 0.037 mol/L ammonia water (see Reagent preparation section). The ammonia water step is not required when using DakoCytomation's Hematoxylin (code S3302).

Note: The use of DakoCytomation's Hematoxylin (codes S3302) or DakoCytomation's Automation Hematoxylin (code S3301) is strongly recommended. Using a 5-minute incubation, this counterstain produces a mild purple/blue end product that does not obscure specific immunostaining. Strong counterstaining may mask weak EGFR expression.

STEP 3. Rinse *gently* in a reagent-quality water bath for 2–5 minutes.

Mounting

Mounting media such as DakoCytomation's Faramount Aqueous Mounting Medium, Ready-to-use (code S3025) or DakoCytomation's Glycergel Mounting Medium (code C0563) is recommended for aqueous mounting. Liquify Glycergel by warming to approximately 40(±5)°C prior to use.

Non-aqueous, permanent mounting is also suitable such as DakoCytomation's Ultramount (code S1964).

Note: Slides may be read when convenient. However, some fading may occur if slides are coverslipped with an aqueous mounting medium and exposed to strong light. To minimize fading, store slides in the dark at room temperature (20–25°C).

Quality control

Deviations in the recommended procedures for tissue fixation, processing and embedding in the user's laboratory may produce significant variability in results, necessitating regular performance of in-house controls in addition to the DakoCytomation-supplied Control Slides. In the USA, consult the quality control guidelines of the College of American Pathologists (CAP) Certification Program for Immunohistochemistry. See also NCCLS Quality Assurance for Immunocytochemistry Approved Guideline,[19] and References 20–23 for additional information.

EGFR pharmDx Control Slides (provided)
Each of the supplied Control Slides contains two pelleted, formalin-fixed, paraffin-embedded human cell lines with staining intensity scores of 2+ and 0. One slide should be stained in each staining procedure. The evaluation of the DakoCytomation-supplied Control Slide cell lines indicates the validity of the staining run. They should not be used as an aid in interpretation of patient results.

Positive Control Tissue
Controls should be fresh autopsy/biopsy/surgical specimens fixed, processed and embedded as soon as possible in the same manner as the patient sample(s). Positive tissue controls are indicative of correctly prepared tissues and proper staining techniques. One positive tissue control for each set of test conditions should be included in each staining run.

The tissues used for the positive tissue controls should give weak positive staining so they can detect subtle changes in the primary antibody sensitivity. The Control Slides supplied with this system or specimens processed differently from the patient sample(s) validate reagent performance only and do not verify tissue preparation.

Known positive tissue controls should only be utilized for monitoring the correct performance of processed tissues and test reagents, NOT as an aid in formulating a specific diagnosis of patient samples. If the positive tissue controls fail to demonstrate appropriate positive staining, results with the test specimens should be considered invalid.

Normal cell types that may be used as a weak EGFR positive include glandular epithelial cells of the prostate and normal lung bronchial epithelium. Because the EGFR staining pattern is heterogeneous, weakly staining normal tissue elements can also be negative. A strong positive internal tissue control is perineurium.

Negative Control Tissue
Use a negative control tissue (known to be EGFR protein negative) fixed, processed and embedded in a manner identical to the patient sample(s) with each staining run to verify the specificity of the primary antibody and to provide an indication of specific background staining. The variety of different cell types present in most tissue sections offers internal negative control sites (this should be verified by the user).

If specific staining occurs in the negative control tissue, results with the patient specimens should be considered invalid. Tissue elements which may be used as negative controls include cardiac myocytes of the heart. Pancreatic acinar cells are also EGFR negative, whereas pancreatic bile duct epithelium stains positively. Lymphocytes when present do not stain with EGFR pharmDx and can be used as a negative internal control.

Nonspecific Negative Control Reagent
Use the supplied Negative Control Reagent in place of the primary antibody with a section of each patient specimen to evaluate nonspecific staining and allow better interpretation of specific staining at the antigen site.

The incubation period for the Negative Control Reagent should correspond to that of the primary antibody.

Assay Verification
Prior to initial use of an antibody or staining system in a diagnostic procedure, the user should verify the antibody's specificity by testing it on a series of in-house tissues with known IHC performance characteristics representing known positive and negative tissues. Refer to the quality control procedures previously outlined in this section of the product insert and to the quality control requirements of the CAP Certification Program for Immunohistochemistry and/or NCCLS Quality Assurance for Immunocytochemistry, Approved Guideline.[19] These quality control procedures should be repeated for each new antibody lot, or whenever there is a change in assay parameters. Carcinomas with known EGFR protein staining intensities and negative tissues are suitable for assay verification.

Table 1: The Purpose of Daily Quality Control

Tissue: Fixed and Processed Like Patient Sample	*Specific Antibody & Detection System*	*Background: Non-specific Antibody (Mouse IgG_1 Negative Control Reagent) provided*
DakoCytomation-supplied Control Slide.	Controls staining procedure only. The evaluation of the DakoCytomation-supplied Control Slide cell lines indicates the validity of the staining run.	
Positive Control: Tissue or cells containing target antigen to be detected (could be located in patient tissue). The ideal control is weakly positive staining tissue to be most sensitive to antibody or antigen degradation.	Controls all steps of the analysis. Validates reagents and procedures used for EGFR staining.	Detection of nonspecific background staining.
Negative Control: Tissues or cells expected to be negative (could be located in patient tissue or positive control tissue).	Detection of unintended antibody cross-reactivity to cells/cellular components.	Detection of nonspecific background staining.
Patient Tissue.	Detection of specific staining.	Detection of nonspecific background staining.

Interpretation of staining procedure

Slide evaluation should be performed by a pathologist using a light microscope. All assessments are to be made on the tumor region of the specimen. For evaluation of the immunocytochemical staining and scoring, an objective of 10X or 20X magnification is appropriate. Use intact cells for interpretation of staining results; necrotic or degenerated cells often stain nonspecifically.[19]

Positive and negative cell lines are included in each EGFR pharmDx kit to validate staining runs, every time the assay is performed. Appropriate staining of the control cell lines provides evidence that the EGFR pharmDx assay is functioning properly. No membrane staining of the CAMA-1 control cell line (0) and moderate brown complete or incomplete membrane staining in the HT-29 control cell line (2+) indicates that the staining run is valid. If the staining intensity of the positive control cell line is too weak or too strong, a false negative or false positive result may be obtained and the test should be repeated. Reference images are available in the EGFR pharmDx Interpretation Guide.

EGFR pharmDx primarily stains cell membranes, demonstrating both complete and incomplete circumferential staining. The immunostaining pattern is frequently heterogeneous, exhibiting various staining intensities within a single neoplasm. Staining has also been observed in the cytoplasm and extracellular spaces.

EGFR pharmDx test results should be reported as positive or negative, using membrane staining as the evaluable structure. Positivity for EGFR expression is defined as any membrane staining above background level, whether or not completely circumferential. Absence of staining should be reported as negative.

Cytoplasmic staining is commonly seen, however the test should be repeated if significant cytoplasmic staining makes it difficult to distinguish membrane staining and interpret the results. Depending on the incubation length and potency of the hematoxylin used, counterstaining will result in a pale to dark blue coloration of the cell nuclei. Excessive or incomplete counterstaining may compromise interpretation of results.

Table 2: EGFR pharmDx Staining Results

Report to treating physician	Definition	
EGFR negative	Absence of specific membrane staining within the tumor	
EGFR positive	Positive (1+) staining is defined as any IHC staining of tumor cell **membranes** above background level; whether it is complete or incomplete circumferential staining.	
	Staining intensity	Percent of tumor cells staining
	1+, 2+ or 3+	≥ 1%

Limitations

General Limitations
IHC is a multistep diagnostic process that requires specialized training in the selection of the appropriate reagents; tissue selection, fixation, and processing; preparation of the IHC slide; and interpretation of the staining results.

Tissue staining is dependent on the handling and processing of the tissue prior to staining. Improper fixation, freezing, thawing, washing, drying, heating, sectioning, or contamination with other tissues or fluids may produce artifacts, antibody trapping, or false-negative results. Inconsistent results may be due to variations in fixation and embedding methods, or to inherent irregularities within the tissue.

Excessive or incomplete counterstaining may compromise proper interpretation of results.

The clinical interpretation of any positive staining or its absence must be evaluated within the context of clinical presentation, morphology and other histopathological criteria. The clinical interpretation of any staining, or its absence must be complemented by morphological studies and proper controls as well as other diagnostic tests. It is the responsibility of a qualified pathologist who is familiar with the antibodies, reagents and methods used to interpret the stained preparation. Staining must be performed in a certified licensed laboratory under the supervision of a pathologist who is responsible for reviewing the stained slides and assuring the adequacy of positive and negative controls.

Tissues from persons infected with hepatitis B virus and containing hepatitis B surface antigen (HBsAg) may exhibit nonspecific staining with horseradish peroxidase.[24]

Reagents may demonstrate unexpected reactions in previously untested tissues. The possibility of unexpected reactions even in tested tissue groups cannot be completely eliminated due to biological variability of antigen expression in neoplasms, or other pathological tissues.[22] Contact DakoCytomation technical support with documented unexpected reactions.

False-positive results may be seen due to non-immunological binding of proteins or substrate reaction products. They may also be caused by pseudoperoxidase activity (erythrocytes) and endogenous peroxidase activity (cytochrome C).[21]

Note: The reagents and instructions supplied in this system have been designed for optimal performance. Further dilution of the reagents or alteration of incubation times or temperatures may give erroneous or discordant results.

Product Specific Limitations
False-negative results could be caused by degradation of the antigen in the tissues over time. Specimens should be stained within 2 months of mounting of tissues on slides when stored at room temperature (20–25°C).[14,25]

For optimal and reproducible results, the EGFR protein requires proteolytic digestion when tissues are routinely fixed (neutral buffered formalin) and paraffin embedded.

Performance characteristics

Specificity
The EGFR antibody, clone 2-18C9 (2-18C9) has been tested for reactivity against cell lines expressing EGFR, HER2, HER3 and HER4. In Western blots of SKBR3 and A431 cell lysates, 2-18C9 recognized a 170 kD band which is consistent with the known molecular weight of EGFR. Clone 2-18C9 has also been found to recognize the EGFRvIII (145 kD) form of the receptor in immunohistochemistry, flow cytometry and Western blotting of EGFRvIII transfected cell lines. In Western blotting experiments, 2-18C9 was unreactive with HER2 positive CAMA-1 cell lysates, HER3-transformed *E. coli* BL-21 protein extracts and CHO-HER4 transfected cell lysates. Additionally, Chinese Hamster Ovary (CHO) transfectants expressing myc (vector tag), either alone or coexpressed with one of the HER family members, were grown in chamber slides that were formalin-fixed and paraffin-embedded, and stained with anti-myc and 2-18C9. The myc antibody stained all five CHO transfectants, whereas 2-18C9 only stained the CHO cells transfected with HER1.

Clinical trials
Three colorectal carcinoma (CRC) cetuximab drug trials (EMD 62202-007, IMCL CP02-9923 and IMCL CP02-0141) were performed in which DakoCytomation EGFR positive immunohistochemistry (IHC) staining test results were used as one of the criteria for study eligibility. In 2 of 3 studies including the pivotal trial (EMD 62202-007), the threshold for positivity was set at 1+ out of a possible 0 to 3+ for staining intensity and 1% staining of the total tumor cells. This threshold was selected because subgroup analysis of a full range

of different thresholds of membrane staining criteria and other differentiating criteria could detect no significant difference in clinical outcome.

Pivotal trial
In the pivotal trial (EMD 62202-007), patients with EGFR pharmDx positive test results were treated with cetuximab in combination with irinotecan or with cetuximab alone. 577 tumor specimens were tested. 474/577 (82%, 95% CI = 78.1%, 86.1%) of the CRC specimens tested were positive for EGFR pharmDx expression. 329 EGFR pharmDx patients were available for 2:1 randomization to the two arms of the pivotal drug trial. In this trial, patients who received irinotecan plus cetuximab achieved a response rate of 50/218 (22.9%, 95% CI = 17.5%, 29.1%). Patients who received cetuximab alone achieved a response rate of 12/111 (10.8%, 95% CI = 5.7%, 18.1%). Only DakoCytomation EGFR positive persons received cetuximab treatment. The response rate for EGFR negative persons is unknown, and therefore cannot be compared. There was no correlation between the degree of tumor response and the percentage of EGFR-positive cells or EGFR-staining intensity. (See Table 3)

Table 3: Cetuximab Pivotal Trial (EMD 62202-007) Response Rates

	Total number of patients tested	*Response Rate# of cases treated with cetuximab and irinotecan*	*Response Rate# of cases treated with cetuximab alone*
EGFR pharmDx +	474*	50/218 (22.9%, 95% CI = 17.5%, 29.1%)	12/111 (10.8%, 95% CI = 5.7%, 18.1%)
EGFR pharmDx -	103	None treated	None treated

*329 Patients were available for 2:1 randomization to the two arms of the drug trial

Response rate was the proportion of patients in the entire study population with a decrease by >/=50% in the sum of the perpendicular diameter of all measurable tumor (i.e., a 50% or more decrease in tumor by surface area) that persisted for at least 28 days.

Supportive Studies
The EGFR pharmDx assay was used to enroll patients in the pivotal trial (EMD 62202-007) and one supportive study (IMCL CP02-0141) during cetuximab development. In study IMCL CP02-0141, 140 specimens were tested. Of these specimens, 105 /140 (75%, 95% CI = 66.9%, 83.1%) specimens had tumors that were identified as EGFR pharmDx positive. A total of 61 patients were enrolled in this study; 57 patients received cetuximab.

In an additional supportive study (IMCL CP02-9923), a prototype EGFR pharmDx kit (composed of the same primary antibody and detection system as above), was used to enroll patients. A total of 412 specimens from 401 patients were tested. 292/401 (72.8%, 95% CI = 68.0%, 77.6%) patients had a positive test result. 139 patients were enrolled; 138 received cetuximab plus irinotecan.

Table 4: Summary of EGFR Percent Positivity in Colon Cancer Patients

Study ID	Positive Ratio (# positive/# tested)	% Positive	95% Confidence Intervals
Pivotal Trial EMD 62202-007	474/577	82.1%	78.1 – 86.1%
Supportive Study IMCL CP02-0141	105/140	75.0%	66.9 – 83.1%
Prototype EGFR Study IMCL CP02-9923	292/401	72.8%	68.0 – 77.6%

Reproducibility
Inter-run reproducibility
Inter-run reproducibility was tested using manual methodology at two laboratories by two technicians in each laboratory over 3 days with 5 different specimens (4 positive, 1 negative in each lab), of different staining intensity scores randomized and masked. Excellent reproducibility (100%) was seen for positive versus negative results (0 vs. 1+, 2+ and 3+). Staining intensity varied by 1+ in two of the positive specimens and by 2+ in one specimen (in one of the tests, the positive tissue element was mostly washed off the slide). The two negative specimens remained negative.

Inter-laboratory reproducibility of staining
Inter-laboratory reproducibility was tested at three geographically separated laboratories with 30 randomized and masked specimens of various IHC staining intensity scores. Freshly cut slides were forwarded to each testing laboratory for manual and automated staining and evaluation by a pathologist. Inter-laboratory percent agreement was 100% for a dichotomous positive/negative determination where 0 was negative and 1+, 2+ and 3+ were positive for EGFR protein expression for both manual and automated testing procedures.

Immunoreactivity

A summary of the EGFR pharmDx immunoreactivity on the recommended panel of normal tissues is presented in Table 5. All tissues were formalin-fixed and paraffin-embedded.

Table 5: Evaluation of Normal Tissue Staining by EGFR pharmDx*

Tissue Type (# tested)	*Positive Tissue Element Staining and Staining Pattern*
Adrenal (2)	Cortical cells (2+): Cytoplasmic
Bone Marrow (3)	None
Breast (2)	Lobular epithelial cells (2+): Membrane and cytoplasmic
Brain/Cerebellum (3)	Molecular layer (1+): Extracellular
Brain/Cerebrum (3)	None
Cervix (3)	Basalar squamous epithelial cells (2+): Membrane
Colon (3)	None
Esophagus (2)	Basalar squamous epithelial cells (2+): Membrane
Heart (3)	None
Kidney (3)	Tubules (1+): Cytoplasmic staining (granular)
Liver (3)	Hepatocytes (sinusoids) (3+); Bile ducts (3+): Membrane and cytoplasmic
Lung (3)	Alveolar lining cells/ basalar bronchial cells (myoepithelial cells) (2+): Membrane and cytoplasmic
Mesothelial Cells (3)	Mesothelial cells (2+): Membrane and cytoplasmic
Ovary (3)	None
Pancreas (3)	Ducts (2+): Membrane
Parathyroid (1)	None
Peripheral Nerve (3)	Nerve cell processes (1+): Fibrous
Pituitary (3)	None
Prostate (3)	Glandular epithelial cells (2+): Membrane
Salivary Gland (3)	Ductal elements (1+): Cytoplasmic
Skeletal Muscle (3)	None
Skin (3)	Squamous cells, adnexal structures (2+): Membrane and cytoplasmic
Small Intestine (3)	None
Spleen (3)	None
Stomach (3)	None
Testis (3)	None
Thymus (3)	None
Thyroid (3)	None
Tonsil (3)	Squamous epithelium (3+): Membrane and cytoplasmic
Uterus (3)	Endometrial gland epithelium (2+): Membrane and cytoplasmic Endometrial stromal cells (2+): Membrane and cytoplasmic Myometrium: None

*The majority of tissues tested had positive staining of fibroblasts in stromal tissue (1+, fibrous) as well as perineural fibroblasts and myoepithelial cells. Endogenous peroxidase-induced staining of eosinophils has been observed occasionally.

Table 6: Troubleshooting

Problem	*Probable Cause*	*Suggested Action*
1. No staining of slides.	1a. Reagents not used in proper order.	1a. Review application of reagents.
	1b. Sodium azide in wash buffer bath.	1b. Use fresh, azide-free wash buffer provided in the kit.
2. Weak staining of slides.	2a. Inadequate Proteinase K proteolytic digestion.	2a. Verify that the Proteinase K solution is incubated for a full 5 minutes.
	2b. Sections retain too much solution after wash bath.	2b. Gently tap off excess solution before wiping around section.
	2c. Slides not incubated long enough with antibodies or DAB+ Substrate-Chromogen Solution.	2c. Review recommended incubation times.
	2d. Inappropriate fixation method used.	2d. Ensure that patient tissue is not over-fixed and that an approved fixative is being used. (See Specimen preparation section)
3. Excessive background staining of slides.	3a. Paraffin incompletely removed.	3a. Use fresh cleaning solutions and follow procedure outlined in the Deparaffinization and Rehydration section.
	3b. Starch additives used in mounting sections to slides.	3b. Avoid using any additives for adhering sections to glass slides. Many of these are immunoreactive.
	3c. Slides not thoroughly rinsed.	3c. Use fresh solutions in buffer baths and wash bottles.
	3d. Sections dried during staining procedure.	3d. Use humid chamber. Verify that the appropriate volume of reagent is being applied to slides. Wipe only 3 to 4 slides at a time before applying reagent.
	3e. Nonspecific binding of reagents to tissue section.	3e. Check for proper fixation of the specimen and/or the presence of necrosis.
4. Tissue detaches from slides.	4a. Use of incorrect slides.	4a. Use charged (Fisher's SuperFrost Plus) or silanized slides such as DakoCytomation's Silanized Slides (code S3003).
5. Excessively strong specific staining.	5a. Inappropriate fixation method used.	5a. Ensure that only approved fixatives and fixation methods are used. (See Specimen preparation section)
	5b. Reagent incubation times too long.	5b. Review Staining Protocol instructions.
	5c. Inappropriate wash solution used.	5c. Use only the diluted wash buffer that is supplied with the kit.
6. Weak staining of the 2+ control slide cell line.	6a. Inadequate Proteinase K proteolytic digestion.	6a. Verify that the Proteinase K solution is incubated for a full 5 minutes.
	6b. Slides not incubated long enough with antibodies or DAB+ Substrate-Chromogen Solution.	6b. Review recommended incubation times.
	6c. Degradation of Control Slide.	6c. Check expiration date and kit storage conditions printed on the package label.
7. Overdigestion of tissue.	7a. Overincubation with Proteinase K.	7a. Verify that the Proteinase K solution is incubated for no more than 5 minutes.
	7b. Inadequate fixation.	7b. Use post-fixation procedure listed under staining protocol on new slides and stain using existing procedure.

Note: Refer also to the Troubleshooting section in the DakoCytomation *Handbook: Immunochemical Staining Methods*, 3rd Edition,[16] the *Atlas of Immunohistology*,[23] or *Immunoperoxidase Techniques. A Practical Approach to Tumor Diagnosis*.[20] Contact DakoCytomation Technical Support to report unusual staining.

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N — Dangerous to the Environment French Translation German Translation	O — Oxidizing French Translation German Translation	T+ — Very Toxic French Translation German Translation	Xn — Harmful French Translation German Translation	F+ — Extremely Flammable French Translation German Translation

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Manufacturer Fabricant Hersteller	LOT — Batch Code Code du lot Chargenbezeichnung	Σ — Contains Sufficient for <N> Tests French Translation German Translation	EC REP DakoCytomation Denmark A/S Produktionsvej 42 DK-2600 Glostrup Denmark Tel +45 4485 9500 Fax +45 4485 9595 www.dakocytomation.com
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